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Fungicides Influence Growth and Development of Specific Ectomycorrhizae on Loblolly Pine Seedlings

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ABSTRACT. Four fungicides—captan, benomyl, PCNB, and benodanil—in single (1×) and double (2×) applications were used as soil drenches on fumigated nursery soil infested with *Pisolithus tinctorius* or *Thelephora terrestris* after sowing seed of *Pinus taeda*. Control soil was naturally colonized.

Seedlings were lifted 8 months after sowing. Ectomycorrhizal development on seedlings and basidiocarp production by *P. tinctorius* was significantly greater than fungicide-free controls in plots treated with benomyl and captan at either rate and less in plots treated with benodanil; PCNB had no effect. Ectomycorrhizal development by *T. terrestris* was greatest in plots with 2× rates of benomyl and captan. PCNB and benodanil had no effect on *Thelephora* development but both fungicides decreased basidiocarp production. Considerable seedling mortality occurred shortly after applications of PCNB, which reduced plot density and resulted in larger seedlings in those plots. The incidence of fusiform rust galls was reduced by benodanil in all fungus treatments.

In a concurrent laboratory study of test fungi on agar medium, *P. tinctorius* did not grow on any concentration of bendodanil or on 2× or 4× rates of PCNB. Mycelial growth was strongly inhibited by PCNB at 0.5× and at the 1× rate, and slightly inhibited by benomyl at 2× and 4× application rates; captan had no effect on mycelial growth. Benodanil completely inhibited mycelial growth of *T. terrestris* but growth was only slightly inhibited by benomyl at the 4× rate; captan and PCNB had no effect. FOREST SCI. 27:167–176.

ADDITIONAL KEY WORDS. *Cronartium quercuum* f. sp. *fusiforme*, fungicide-nontarget organisms, seedling quality, *Pinus taeda*.

THE IMPORTANCE OF ECTOMYCORRHIZAE to survival and growth of tree seedlings is well documented. In many parts of the world, special cultural procedures are used in tree nurseries to encourage ectomycorrhizal development (Mikola 1973, Bowen and Theodorou 1973). Procedures include the addition of soil containing these fungi, maintenance of high organic matter and low to moderate fertility,

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NOTE: This paper reports research involving pesticides. It does not contain recommendations for their use, nor does it imply that the uses discussed here have been registered. All uses of pesticides must be registered by appropriate State and/or Federal agencies before they can be recommended.

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and growing tree hosts near the nursery to encourage production of fruiting bodies for natural colonization.

Considerable research in Austria, Argentina, Australia, and the United States has been aimed at introducing pure cultures of fungi into nursery soil whose ectomycorrhizae are more beneficial to tree seedlings after outplanting than are those of naturally occurring nursery fungi (Marx 1980). In the United States, seedlings of various southern pines have been successfully tailored with ectomycorrhizae formed with pure cultures of *Pisolithus tinctorius* (Pers.) Coker and Couch, and *Thelephora terrestris* Ehrh. ex Fr. in different nurseries (Marx and others 1976, Marx and Artman 1978, Marx and others 1978). Pine seedlings with *Pisolithus* ectomycorrhizae survive and grow better than seedlings with ectomycorrhizae formed in the nurseries by naturally occurring fungi, such as *T. terrestris,* on adverse soils (Marx and Artman 1979, Berry and Marx 1978) and on routine reforestation sites (Marx and others 1977) in the southern United States. Following these field successes, numerous tests are now underway to determine the biological and economic feasibility of commercially producing inoculum of *P. tinctorius* for use in forest tree nurseries.

Many different fungicides are used to control disease in the production of nursery seedlings (Peterson and Smith 1975), and many of them affect ectomycorrhizal development. Bakshi and Dobriyal (1970) found that captan and PCNB used to control damping-off in nurseries in India delayed the development of naturally occurring ectomycorrhizae on seedlings of *Pinus patula* until the fungicides had lost their effectiveness. In Malaysia, Hong (1976) observed that ectomycorrhizal development on *Pinus caribaea* seedlings was suppressed by chlorothalonil and thiram and stimulated by captafol and captan, whereas benomyl had no effect. Laiho and Mikola (1964) reported that PCNB inhibited growth of several ectomycorrhizal fungi in pure culture but did not alter development of naturally occurring ectomycorrhizae of pine and spruce seedlings when they were applied to soils in Finnish nurseries. In nursery and greenhouse tests in Australia, Theodorou and Skinner (1976) found that seedcoat dressings of captan, zineb, and thiram inhibited ectomycorrhizal development of *Pinus radiata* seedlings grown from seed inoculated with basidiospores of different fungi. The fungicides, applied in this manner, apparently killed the test fungi but had no effect on the subsequent development of ectomycorrhizae formed by naturally occurring fungi. Kelley (1979) reported that benodanil, a systemic fungicide under test for control of fusiform rust on pines in the southern United States, prevented pure culture growth of several ectomycorrhizal fungi and when applied as a drench to nursery soil it delayed development of naturally occurring ectomycorrhizae on *Pinus taeda* L. Recently, Pawuk and others (1980) found that the development of *Pisolithus* ectomycorrhizae on *Pinus palustris* seedlings grown for 14 weeks in a pine bark medium in containers was completely inhibited by PCNB, reduced by captan and fenaminosulf, and stimulated by benomyl and Banrot. Thiabendazole and Truban had no effect.

Because of these known effects of certain fungicides, those that are currently used or are being considered for use to control diseases in forest nurseries should be evaluated to determine their effect on *Pisolithus* ectomycorrhizal development before decisions are made on the practical application of *P. tinctorius* to forestation programs. The main objective of this study was to determine the effect of single and double applications of captan (cis-N-(trichloromethyl) thio)-4-cyclohexene-1,2 dicarboximide), benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate), PCNB (pentachloronitro-benzene), and benodanil (2-iodobenzanilide) on ectomycorrhizal development by pure cultures of *P. tinctorius, T. terrestris,* and by naturally occurring fungi on seedlings of *P. taeda* in fumigated nursery soil. A secondary objective was to determine the effect of these fungicides

on pure culture growth of the two fungi. *Thelephora terrestris* was included in these tests because it is naturally occurring in tree nurseries throughout the United States and abroad; information on its reaction to these fungicides may be valuable for its future management in nurseries.

Materials and Methods

Nursery Study.—Mycelial inoculum of *Pisolithus tinctorius* (isolate 227) and *Thelephora terrestris* (isolate 223) was grown at room temperature in 2-liter glass jars containing vermiculite and peat moss moistened with nutrients (Marx and Bryan 1975). After 4 months, the contents of the jars were removed, wrapped in cheesecloth, leached thoroughly in tapwater, and squeezed by hand to remove excess water. The leached inocula were placed 5 to 6 cm deep on steel screens (16 mesh) framed with wood and dried at 20° to 24°C and 35 to 45 percent relative humidity. The layer of inocula on the screens was turned by hand every 3 to 4 h for the 60-h drying period to minimize excessive drying of the surface particles. Final bulk density of the *Pisolithus* inoculum was 350 g/l with 27 percent moisture. Bulk density of the *Thelephora* inoculum was 386 g/l with 50 percent moisture. Control inoculum was fungus-free-vermiculite-peat-nutrient medium leached and dried to a bulk density of 320 g/l with 18 percent moisture. The original volume of inoculum contained in the jars was reduced nearly 55 percent by the leaching and drying process. Dried inoculum was packaged in 400 ml volumes in plastic bags and stored for 4 days at 5°C until used.

One hundred and thirty-five wood-frame microplots (0.6 × 0.6 × 0.6 m) were placed 1.5 m apart on level ground in five blocks of 27 microplots each. Each microplot was filled to a depth of 20 cm with gravel and fumigated with methyl bromide. A soil mixture of forest clay loam, sand, and milled pine bark (2:1:1 v:v:v) was fumigated on a concrete pad and then used to fill the microplots. All fumigation was done with Dowfume MC-2 (Dow Chemical Co., Midland, Mich.) at 1 kg/18 m^2 of soil surface (25 cm deep) under clear polyethylene plastic for 48 h. During fumigation, soil moisture was approximately 50 percent of field capacity and soil temperatures ranged from 26° to 38°C. The soil mixture after fumigation had a pH 5.4 and contained 6 μg/g of available P, and 60, 110, 20, and 40 μg/g of exchangeable K, Ca, Mg, and Mn, respectively. Total N was 140 μg/g and organic matter was 2.1 percent.[1]

Inoculum at a rate of 1.08 liter/m^2 of soil surface (400 ml/microplot) and granulated commercial 10-10-10 fertilizer at 300 kg/ha were broadcast evenly over the soil in microplots and mixed into the upper 10 to 12 cm of soil. Fertilizer and control inoculum were similarly added to soil of control plots. Nine of the 27 microplots in each block were randomly selected for infestation with inoculum of either *Pisolithus, Thelephora,* or the control. Stratified seeds of *Pinus taeda* (mixed lot from piedmonts of Mississippi and South Carolina) were treated with thiram-latex sticker. One hundred and twenty seeds were evenly distributed in four rows 0.6 m long spaced 12 cm apart in each microplot and covered with 5 mm of soil. Fumigated pine straw was placed 2 cm deep over the soil as a mulch.

Captan (50 percent WP) at 4.5 kg a.i./ha, benomyl (50 percent WP) at 11.2 kg a.i./ha, PCNB (75 percent WP) at 6.7 kg a.i./ha, or benodanil (BAS-317-10F emulsified concentrate) at 15.3 kg a.i./ha were mixed in water and applied as a drench (2 liter/microplot) to two random microplots per fungus treatment in each block 9 days after sowing (1×) and to one of these microplots again 2 weeks later

[1] Soil analyses were done by Carol G. Wells, USDA Forest Service, Forestry Sciences Laboratory, Research Triangle Park, NC 27709.

(2×). The remaining nontreated microplot per fungus per block was a fungicide control and it received only water. The experimental design was a randomized complete block with five blocks each containing 27 treatments: 9 fungicide treatments (four fungicides at two application frequencies and a fungicide-free control) and 3 ectomycorrhizal fungus treatments (*Pisolithus*, *Thelephora*, or noninoculated).

The study was installed in late April 1978. Seedlings were irrigated twice weekly and broadcast fertilized with 56 kg of N/ha (as NH_4NO_3) in early July and again in early August. Seedling foliage was sprayed weekly from mid-June through mid-September with dimethoate at the recommended label rate to control insects. Microplots were examined twice weekly beginning in late July and the incidence of basidiocarps of *P. tinctorius* and *T. terrestris* was recorded. Basidiocarps of *P. tinctorius* were removed; those of *T. terrestris* were not, since removal would have caused physical damage to the succulent seedlings.

In August a midstudy evaluation was made by removing five random seedlings per microplot and visually assessing roots for ectomycorrhizal development. Following seedling dormancy in early November, roots were vertically cut between rows and undercut 20 cm deep with a shovel. Seedlings were removed by hand and the roots washed in water. All seedlings were examined for specific ectomycorrhizae and rust galls caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* Burdsall and Snow, and were graded. Seedlings with less than 12 cm height, 2 mm root collar diameter, without secondary needles, or with rust galls were considered culls and discarded; the remaining seedlings were considered plantable. Ten plantable seedlings per microplot were selected at random and measured for height, root collar diameter, and top and root fresh weight. Ectomycorrhizal development was visually estimated, without magnification, as a percentage of feeder roots (Marx and Bryan 1975). Data were evaluated by analysis of variance and significant means were separated with Duncan's Multiple Range Test ($P = 0.05$).

Laboratory Study.—Modified Melin-Norkrans (MMN) agar medium (Marx 1969) with glucose was prepared, autoclaved, and placed in a 52°C water bath. Nonsterile aqueous suspensions of the fungicides were added to the medium to obtain concentrations of each fungicide equal to the single (1×), double (2×), half the single (0.5×), and twice the double (4×) application rates used in the nursery test. The suspensions were thoroughly mixed in the medium and poured into petri dishes (30 ml/dish). The pH of the agar medium containing the 16 fungicide treatments varied from pH 5.6 to 5.8; control MMN agar medium without fungicide was pH 5.4. The test fungi were grown for 20 days in dishes of MMN agar medium at 25°C. Eight-mm diameter mycelial-agar discs were removed from the outermost periphery of mycelial growth and placed (mycelium down) on the amended MMN medium. Eight dishes per fungus-fungicide-concentration treatment were prepared and incubated at 25°C. Diameters of mycelial colonies were measured after 9 days for *P. tinctorius* and 14 days for *T. terrestris*; data were analyzed as described earlier.

Results

Nursery Study.—Midstudy evaluation revealed that the 1× and 2× benodanil treatments were inhibiting ectomycorrhizal development and basidiocarp production by both test fungi, particularly *Pisolithus*. Other fungicides had no apparent effect on ectomycorrhizal development or basidiocarp production of either test fungus at this time.

It was obvious at midstudy and at final evaluation that the prevalence of naturally occurring ectomycorrhizae on seedlings was low. A late spring–early sum-

mer drought probably limited basidiocarp production in forests adjacent to the nursery and, consequently, limited basidiospore colonization of the fumigated soil. At midstudy time, only 4 to 12 percent of feeder roots had naturally occurring ectomycorrhizae on seedlings in noninoculated, fungicide-free plots; in past studies in this nursery, control seedlings usually had 40 percent by August.

The naturally occurring ectomycorrhizae were monopodial to simple coralloid, dark brown, with smooth fungus mantles. They were easily distinguished from the complex coralloid, mustard-yellow, pitted-mantled ectomycorrhizae formed by *Pisolithus* and the complex coralloid, white to light brown, smooth-mantled ectomycorrhizae formed by *Thelephora*. Both test fungi produced abundant hyphal strands on and around seedling roots but hyphal strands were not detected on roots or in soil of seedlings in noninoculated, control plots. The naturally occurring ectomycorrhizal fungi in noninoculated plots were not identified because fruiting bodies were absent.

Seedling heights varied from 19.0 to 22.5 cm and were not affected by fungus or fungicide treatments. Other seedling growth parameters were affected by these treatments (Table 1). Differences in total fresh weight of seedlings were found between PCNB treatments and controls. These differences were apparently due to fewer seedlings in the PCNB plots and not to ectomycorrhizal fungus treatments. PCNB caused significant seedling mortality during May, which resulted in fewer seedlings per plot.

The fungicides significantly affected ectomycorrhizal development by both test fungi (Table 1). Captan and benomyl at the 1× and 2× rates increased development of *Pisolithus* ectomycorrhizae and production of basidiocarps, whereas benodanil decreased them; PCNB had no affect. Captan and benomyl at the 2× rate increased development of *Thelephora* ectomycorrhizae. The other fungicides had no effect on *Thelephora* ectomycorrhizal development, but benodanil at the 1× and 2× rates decreased the incidence of *Thelephora* basidiocarps. Benodanil treatments also reduced the percentage of seedlings which had ectomycorrhizae formed by *Pisolithus* and *Thelephora*. More naturally occurring ectomycorrhizae were observed on seedlings treated with both benomyl treatments than on control seedlings without fungicides.

Generally, seedlings in plots receiving *Pisolithus* inoculum, irrespective of fungicide treatments, were larger with a lower cull percentage than seedlings in *Thelephora* or noninoculated control plots. This observation was not related to seedling densities since there was an overall average of 85 seedlings per plot in each of the three fungus groups. The total number of seedlings per plot was greater and there were fewer cull seedlings in the *Pisolithus* and *Thelephora* plots treated with benomyl at the 2× rate than there were in noninoculated plots receiving the same fungicide treatments.

The incidence of fusiform rust galls on seedlings ranged from 0.1 to 4.1 percent, an infection rate unusually low for this nursery. In past years, loblolly pine seedlings in this nursery usually averaged a 25-percent infection rate. Apparently the early season drought was responsible for this low incidence of rust. Although statistical differences were found between fungicide treatments and controls, particularly with benodanil, the low incidence of the disease prevented meaningful biological interpretations.

Laboratory Study.—Benodanil, at all concentrations, completely inhibited mycelial growth of both fungi while captan had no affect on either fungus (Table 2). Benomyl at rates of 2× and 4× inhibited growth of *P. tinctorius* while *T. terrestris* was inhibited only by the highest rate. PCNB did not affect growth of *T. terrestris* but all concentrations inhibited growth of *P. tinctorius*.

TABLE 1. Growth, ectomycorrhizal development, and plot parameters of 7-month-old seedlings of loblolly pine in soil infested with Pisolithus tinctorius, Thelephora terrestris, *or naturally occurring fungi and drenched with fungicides 9 days after sowing (1×) and again 2 weeks later (2×).*[1,2]

Soil condition, fungicide and rate[3]	Seedling data				Plot data				
	Root collar diameter	Total fresh weight	Ectomycorrhizae by—		Seedlings with test fungus	Seedlings	Cull seedlings	Fusiform rust	Basidiocarps test fungus
			Test fungus	All fungi					
	mm	*g*	*percent*	*percent*	*percent*	*number*	*percent*	*percent*	*number*
Soil infested with *Pisolithus tinctorius*									
Captan 1×	4.3	11.0	83	84*	100	87	10.5	1.5*	2
Captan 2×	4.2	10.6	82	83*	100	89	12.4	1.6	4
Benomyl 1×	4.1	10.2	80	81*	100	93	9.4	1.3	2
Benomyl 2×	4.1	10.5	83	85*	100	93*	9.6*	1.7	2
PCNB 1×	4.3	12.2	68	71*	100	82	10.7	0.7	2
PCNB 2×	4.7	14.2*	66	68*	100	64	12.2	0.4*	1
Benodanil 1×	4.6	13.8	46	48*	85	79	13.1	0.3	1
Benodanil 2×	4.1	10.6	13	19	47	91	18.3	0.2	0
Control	4.2	11.0	74	75*	100	85	11.9	2.2	2
Overall ×	4.3	11.6	66	68	92	85	12.0	1.1	1.9
Soil infested with *Thelephora terrestris*									
Captan 1×	4.1	10.7	73	74*	100	86	13.7	2.0	19
Captan 2×	4.0	10.5	84	85*	100	82	15.9	1.4	14
Benomyl 1×	3.9	10.0	72	79*	100	94	13.4	1.2	22
Benomyl 2×	3.9	9.9	81	84*	100	97*	14.1*	1.9	25
PCNB 1×	4.0	9.9	61	64*	100	87	11.1	1.5	26

TABLE 1. Continued.

Soil condition, fungicide and rate[3]	Seedling data				Plot data				
	Root collar diameter	Total fresh weight	Ectomycorrhizae by— Test fungus	Ectomycorrhizae by— All fungi	Seedlings with test fungus	Seedlings	Cull seedlings	Fusiform rust	Basidiocarps test fungus
PCNB 2×	4.6	14.2*	58	61*	100	63	10.5	1.1	16
Benodanil 1×	3.9	10.0	62	68*	96	82	15.7	0.1	8
Benodanil 2×	4.0	10.1	52	59*	92	89	26.4	0.3	1
Control	3.7	10.0	69	70*	100	88	19.6	1.9	18
Overall ×	4.0	10.6	68	72	99	85	16.2	1.3	16.6
Soil not infested (control)									
Captan 1×	4.1	10.2	N/A	13	0	87	17.3	4.1	N/A
Captan 2×	4.1	10.7	N/A	13	0	87	13.4	1.0	N/A
Benomyl 1×	4.0	10.3	N/A	19	0	93	16.1	0.7	N/A
Benomyl 2×	3.9	9.9	N/A	22	0	83	23.5	1.7	N/A
PCNB 1×	4.2	11.1	N/A	8	0	79	12.1	0.8	N/A
PCNB 2×	4.1	11.3	N/A	9	0	72	15.6	2.3	N/A
Benodanil 1×	4.1	10.4	N/A	13	0	80	15.4	0.3	N/A
Benodanil 2×	3.8	9.0	N/A	9	0	95	18.2	0.2	N/A
Control	3.9	9.8	N/A	11	0	87	16.3	1.4	N/A
Overall ×	4.0	10.3		13	0	85	15.3	1.4	

[1] Seedling means are from 10 seedlings randomly removed from each of five microplots per treatment; plot means are from all seedlings in each of five microplots per treatment.

[2] Means with broken under line (--) within a fungus treatment differ significantly from fungicide-free controls, and * denotes that means within a fungicide treatment differ significantly from fungus-free controls which received the same fungicide and rate ($P = 0.05$).

[3] 1× and 2× fungicidal rates were: captan 4.5 and 9.0 kg/ha; benomyl 11.2 and 22.4 kg/ha; PCNB 6.7 and 13.4 kg/ha; benodanil 15.3 and 30.6 kg/ha.

TABLE 2. Colony diameter (mm) of Pisolithus tinctorius *and* Telephora terrestris *after 9 and 14 days, respectively, at 25°C on agar medium amended with different concentrations of fungicides. Each value is the mean of 7 to 8 petri-dish cultures per treatment.*

Fungicide	Concentration a.i.	*Pisolithus tinctorius*	*Thelephora terrestris*
	$\mu g/g$[1]		
Captan	1.0	55	38
	2.0	54	39
	3.9	54	39
	8.8	57	39
Benomyl	2.5	52	41
	5.0	48	39
	10.0	36*	36
	20.0	36*	29*
PCNB	1.5	15*	44
	3.0	7*	43
	6.0	0*	39
	12.0	0*	38
Benodanil	3.4	0*	0*
	6.8	0*	0*
	13.7	0*	0*
	27.3	0*	0*
Control	None	53	41

[1] Order of concentrations is equal to 0.5×, 1×, 2×, and 4× recommended concentrations for soil drench applications.

* Denotes means significantly different from controls at $P = 0.05$.

Discussion

A comparison of the results of the laboratory and nursery tests shows that mycelium of *Pisolithus* and *Thelephora* is affected more by fungicides in agar medium than in soil. The limited persistence of these fungicides in soil probably accounts for some of this difference in sensitivity. Lack of leaching, microbial activity by associated microflora, and barriers to fungicide diffusion in agar permits longer exposure at higher concentrations to the hyphae in agar culture than in soil. Due to higher nutrient concentrations in agar medium than in soil, the mycelium grows at a faster rate which would increase the metabolic uptake of the fungicides. Also, hyphae of ectomycorrhizal fungi grown in vermiculite particles as initial inoculum may be protected to a certain extent from high concentrations of fungicides in soil. These differences between nursery and laboratory studies on fungicides indicate the limited value of studies on agar medium that are not supported by studies carried out in soil.

Most factors influencing the formation of ectomycorrhizae affect either the susceptibility of the host or the survival and infective potential of the fungal symbionts (Bowen and Theodorou 1973). Although certain fungicides used in this test may have affected the susceptibility of the pine seedlings to root infection by the fungi, results of other research suggest that the action of fungicides is more likely a direct or indirect effect on the fungi. Our results and those of Kelley (1979) indicate that benodanil directly inhibits vegetative growth of the symbiotic fungi and causes a decrease in ectomycorrhizal development. As pointed out by

Kelley (1978), this type of action by benodanil is an example of a fungicide having a detrimental effect on beneficial, nontarget organisms. However, our results and those of others discussed earlier also indicate that fungicides such as benomyl and captan indirectly stimulate ectomycorrhizal development formed by specific, nontarget fungi. The target organisms of benomyl and captan are primarily Hyphomycetes, Ascomycetes (Bollen and Fuchs 1970), and certain Phycomycetes (Agnihotri 1971). A lower population of these fungi and perhaps other microorganisms may increase the effectiveness of inocula of ectomycorrhizal fungi. A reduction in populations of Hyphomycetes and other fungi in rhizospheres of onion plants by benomyl and captan has been demonstrated (deBertoldi and others 1977). Powell and others (1968) reported that captan and other fungicides temporarily decreased soil populations of *Pythium* in soil of pecan trees and stimulated development of ectomycorrhizae formed by naturally occurring *Scleroderma bovista*. The significance of changes in populations of rhizosphere microbes as an indirect action of fungicides on ectomycorrhizal development by *P. tinctorius* and *T. terrestris* must await later investigations.

These studies show that care must be taken in selection of fungicides used to control diseases on pine seedlings in tree nurseries. The qualitative and quantitative effect of fungicides on the development of ectomycorrhizae should be considered when any fungicide is selected to control specific diseases. Disease-free seedlings lacking adequate ectomycorrhizae may be grown to plantable size in a nursery following fungicide application, but the lack of adequate ectomycorrhizae on such seedlings often will result in substandard survival and growth performance following field outplanting (Marx 1980). The stimulating effect of certain fungicides on ectomycorrhizal development, especially those formed by artificially introduced fungi, should be considered an added benefit to the merits of these fungicides.

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